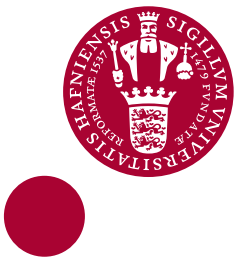


Effects of allochthonous dissolved organic matter input on microbial composition and nitrogen-cycling genes at two contrasting estuarine sites

Happel, Elisabeth Münster; Markussen, Trine Marenda; Teikari, Jonna E.; Huchaiah, Vimala; Alneberg, Johannes; Andersson, Anders F.; Sivonen, Kaarina; Middelboe, Mathias; Kisand, Veljo; Riemann, Lasse

Published in:
F E M S Microbiology Ecology

DOI:
10.1093/femsec/fiz123

Publication date:
2019

Document version
Early version, also known as pre-print

Effects of allochthonous DOM input on microbial composition and nitrogen cycling genes at two contrasting estuarine sites

Elisabeth M. Happel[1], Trine Markussen[1], Jonna E. Teikari[2], Vimala Huchaiah[3], Johannes Alneberg[4], Anders F. Andersson[4], Kaarina Sivonen[2], Matthias Middelboe[1], Veljo Kisand[3], Lasse Riemann[1*]

[1]Marine Biology Section, Department of Biology, University of Copenhagen, Helsingør, Denmark

[2]University of Helsinki, Department of Microbiology, Helsinki, Finland

[3]University of Tartu, Institute of Technology, Tartu, Estonia

[4]KTH Royal Institute of Technology, Science for Life Laboratory, Department of Gene Technology, School of Engineering Sciences in Chemistry, Biotechnology and Health, Stockholm, Sweden

Running title: Effects of DOM on microbial composition and N cycling

*Correspondence: Lasse Riemann, lriemann@bio.ku.dk

Summary

Heterotrophic bacteria are important drivers of nitrogen (N) cycling and the processing of dissolved organic matter (DOM). Projected increases in precipitation will potentially cause increased loads of riverine DOM to the Baltic Sea and likely affect the composition and function of bacterioplankton communities. To investigate this, the effects of riverine DOM from two different catchment areas (agricultural and forest) on natural bacterioplankton assemblages from two contrasting sites in the Baltic Sea were examined. Two microcosm experiments were carried out, where the community composition (16S rRNA gene sequencing), the composition of a suite of N cycling genes (metagenomics), and the abundance and transcription of *amoA* genes (quantitative PCR) were investigated. The river water treatments evoked a significant response in bacterial growth, but effects on overall community composition and on the representation of a suite of N cycling genes were limited. Instead, treatment effects were reflected in the prevalence of specific taxonomic families, specific N related functions, and in the transcription of *amoA* genes. The study suggests that bacterioplankton responses to changes in the DOM pool are constrained to part of the bacterial community, whereas most taxa remain relatively unaffected.

Introduction

Marine heterotrophic bacterioplankton process dissolved organic matter (DOM), thereby mineralizing nutrients essential for growth of phytoplankton and affecting overall productivity in marine waters (Azam *et al.*, 1983). Among nutrients, nitrogen (N) is a primary constituent of various cellular macromolecules, and the availability of N is commonly a limiting factor for primary and secondary production in diverse marine systems (Ryther and Dunstan, 1971; Bristow *et al.,* 2017). In marine coastal systems, the release of dissolved inorganic N (DIN) through the degradation of dissolved organic nitrogen (DON) can be orders of magnitude higher than the input of DIN from land (Knudsen-Leerbeck *et al.*, 2017). Hence, N released or acquired through microbial degradation of nitrogenous DOM is an important N source for bacterioplankton and phytoplankton growth (Bronk *et al.*, 2007).

Bacterioplankton control not only the accessibility of N through DOM processing, but also regulate the oxidative state of N present in the environment through a series of oxidative and reductive processes. Consortia of microorganisms mediate key steps in the marine nitrogen cycle (Falkowski *et al.,* 2008), including e.g. ammonification, nitrogen fixation, nitrification, and denitrification (Zehr and Ward, 2002), and ultimately determine the availability of N for higher trophic levels, e.g. phytoplankton. For instance, the form and oxidation level, e.g. whether inorganic N is available as ammonia or nitrate, may affect both the productivity and the composition of the phytoplankton community (Glibert *et al.,* 2016).

In estuarine environments, riverine DOM is an important source of highly labile N (Seitzinger *et al.,* 2005; Bronk *et al.,* 2007). The characteristics of the riverine DOM may depend on the catchment area and on season. Consequently, it is conceivable that the riverine input,

60 particularly in N limited environments, selects for bacterioplankton capable of hydrolyzing
61 nitrogenous DOM and for taxa involved in down-stream nitrogen cycling processes. Moreover, the
62 bacterial community response would likely rely on the availability and nature of the nitrogenous
63 DOM and depend on bacterioplankton community composition and contemporary environmental
64 conditions. Hence, responses are expected to differ between localities. While these assumptions
65 appear logical, they have to our knowledge not been experimentally verified.

66 The Baltic Sea is the second largest estuarine system in the world and encompasses
67 separate sub-basins with unique geology and a strong north-south salinity gradient driven by river
68 outlets (Rönnberg and Bonsdorff, 2004). The north is characterized by high DOM concentrations
69 and phosphorous (P) limited plankton production whereas the south has lower DOM
70 concentrations and N limited plankton productivity (Bernes, 2005; Rowe *et al.,* 2018). Further,
71 catchment characteristics vary from primarily forest in the north to agricultural landscapes in the
72 south. The gradient in biogeochemistry is also reflected in extensive changes in microbial
73 community composition from north to south (Herlemann *et al.,* 2011). Climate change is predicted
74 to increase precipitation and the allochthonous DOM input via rivers to the Baltic Sea (Andersson
75 *et al.,* 2015). The loading and characteristics of the DOM will likely affect the future microbial
76 community composition, activity, DOM utilization, and nutrient biogeochemistry in the Baltic Sea
77 (Traving *et al.,* 2017; Rowe *et al.,* 2018). However, responses will conceivably vary between north
78 and south due to differences in catchments, characteristics of the incoming DOM, and
79 composition of the recipient microbial communities.

80 In the present study we examined effects of riverine DOM loading in incubation
81 experiments in two contrasting environments; the southern Baltic Sea (Øresund) after the spring

bloom and in the northern Baltic Sea (Storfjärden) in summer (Fig. S1). After the spring bloom, Øresund surface water is typically N depleted whereas the surface water at Storfjärden typically has higher N and DOM concentrations during the summer. We tested the effects of allochthonous DOM loads at both sites by additions of river water from an agricultural and a forest (humic) catchment area, and examined the microbial community composition (16S rRNA genes), the abundance of nitrogen cycling genes (reflecting the metabolic capacities) and the activity of ammonia oxidizers. Since effects on community composition and the composition of functional genes may not be detectable during short-time incubations, we chose to examine changes in functional gene transcription (as a proxy for activity), namely, quantifying the transcription of *amoA* genes (coding for ammonia monooxygenase) - genes involved in nitrification - which is a critical N cycling process facilitating N loss through coupled nitrification-denitrification in the Baltic Sea coastal zone (Hietanen *et al.,* 2012). We anticipated temporal functional succession and treatment-specific responses to our manipulations, and further that effects would differ between the two localities with a more modest response in the relatively DOM and nitrogen-rich northern locality.

Experimental procedures

Experimental setup for Exp I and Exp II

The setup described here was part of a larger experiment reported elsewhere (Markussen *et al.,* 2018). Microcosm experiments with the same setup were conducted at the Marine Biological Laboratory (University of Copenhagen, Denmark) using Øresund water (Exp I) and at the Tvärminne Zoological Field station (University of Helsinki, Finland) using water from Storfjärden,

Gulf of Finland (Exp II). The microcosms consisted of three treatments in triplicates (control in 6 replicates) including control, addition of water from a humic river (hereafter DOMhum) and an agricultural river (DOMagri). Water was collected on April 20th 2015, Øresund (56°3' 26.4" N, 12°38'44.9" E) from 5 m (300 L) and 12 m (10 L) depth, and on July 27th 2015, at Storfjärden, Gulf of Finland (59°51'11.9"N 23°16'19.2"E) 300 L from 0, 5, and 10 m depth (Fig. S1). The water was prefiltered through a 10 m plankton net, filtered through a 0.22 m capsule filter (Optical XL, Millipore), pooled to ensure homogeneity, and filled into microcosms (10 L PC bottles, Nalgene) to represent 60% of the final volume, except for the controls that were filled to represent 80% final volume. The river water was collected 2 days prior to the start of the experiments from the rivers Lapväärti (62°14'20.6"N 21°34'37.5"E, Finland) and Lielupe (56°48'41.6"N 23°35'04.9"E, Latvia; Fig. S1), filtered through a 0.22 m capsule filter (Optical XL, Millipore), and stored at 4°C until use. The salinity of the river water was adjusted with muffled NaCl to in situ levels of 13.4 (Øresund) and 6 (Storfjärden) prior to addition to the microcosms. River water was added to the microcosms representing 20% of the final volume. A plankton inoculum (<10 µm) was added to each batch, representing 20 % of the final volume, to initiate the experiments. The microcosms were incubated in the dark, in a temperature-controlled room at in situ temperature ±3°C. Exp I and Exp II had a duration of 5 and 4 days, respectively. Daily samplings at 09.00 and 21.00 covered a variety of environmental parameters and DNA/RNA sampling.

Bacterial abundance and production, and nutrients

Data were adapted from Markussen *et al.* (2018). Briefly, Samples for bacterial enumeration were fixed with glutaraldehyde (1% final conc., Sigma-Aldrich), stored at -80°C, and later enumerated using SYBR green I (Invitrogen) and a BD FACS Canto II flow cytometer. Bacterial

production was measured by ^{3}H-thymidine incorporation (Fuhrman and Azam, 1982). Ammonium (NH_4^+) concentrations were determined directly from fresh samples using ortho-phthalaldehyde (Holmes *et al.,* 1999) and a rapid flow analyser (Turner Designs Trilogy Laboratory Fluorometer). Nitrate (NO_3^-) and phosphate (PO_4^{3-}) were measured on an auto-analyser according to (Wood *et al.,* 2009) and (Murphy and Riley, 1962), respectively. Dissolved organic carbon (DOC) and nitrogen (DON) was measured on a Shimadzu TOC-L Total Organic Carbon Analyzer (Shimadzu Corporation) as previous described (Paulsen *et al.,* 2017).

DNA and RNA sampling and extraction

Water was sampled in situ, from the inoculum, and twice daily from each microcosm during the experiments. One liter water was collected in 1 L PC bottles (Nalgene), immediately mixed with 100 ml stop-solution (5% phenol in 99.8% ethanol (Khodursky *et al.,* 2003)), and stored for < 24 h at RT in the dark. Fixed samples were then filtered onto 0.22 µm membrane filters (Durapore GVWP04700, Milipore) and stored at -80°C. Nucleic acids were extracted from the filters using the Allprep kit (Qiagen), and then purified and concentrated using the RNA Clean & Concentrator (Zymo) and Genomic DNA Clean & Concentrator (Zymo). DNA and RNA extracts were quantified using Quant-IT RiboGreen and PicoGreen (Invitrogen), respectively.

16S rRNA gene amplicon sequencing and metagenomics

For determining the community composition 16S rRNA genes were amplified from total DNA using the primers Bakt_341F (CCTACGGGNGGCWGCA) and Bakt_805R (GACTACHVGGGTATCTAATCC) (Herlemann *et al.,* 2011), and products were sequenced with on an Illumina MiSeq paired-end multiplex platform at SciLifeLab/NGI (Solna, Sweden). Raw amplicon

reads were quality trimmed (Trimmomatic ver 0.32), and chimeras removed and reads assigned into operational taxonomic units (OTUs) using 97% cut-off in cd-hit-otu (Li *et al.,* 2012). SINA 1.2 (Pruesse *et al.,* 2012) was used against the SILVA database (v. 115) to classify unique OTUs and the relative abundance of each OTU was estimated using an in-house Python script. Sequences were deposited in NCBI SRA (Bioproject number 542 PRJNA435478).

DNA (2-10 ng) from each sample was prepared for metagenomics sequencing with the Rubicon ThruPlex kit (Rubicon Genomics, Ann Arbor, Michigan, USA) according to the instructions of the manufacturer. Cleaning steps were performed with MyOne carboxylic acid-coated superparamagnetic beads (Invitrogen, Carlsbad, CA, USA). Libraries were sequenced on a HiSeq 2500 (Illumina Inc., San Diego, CA, USA). On average, 18 million paired-end reads (125 bp) per sample were generated. Raw reads were quality trimmed with Cutadapt (Martin, 2011) from both read ends and duplicate reads were removed with fastuniq (Xu *et al.,* 2012). High quality reads were mapped on the BARM database containing the most informative reference genomes in the Baltic Sea (Alneberg *et al.,* 2018) with Bowtie2 using default parameters (Langmead *et al.,* 2013). The raw counts were calculated from Bedtools histogram output (Quinlan, 2014) and quantitative abundance of reads were annotated using Clusters of Orthologous Groups (COG) (Galperin *et al.,* 2015).

Reverse transcription and quantification of amoA genes and transcripts

cDNA was synthesized using gene-specific reverse primers and the TaqMan Reverse transcription kit (Life Technologies) according to manufacturer's protocol. The RT products from each sample were quantified using PicoGreen and used to calculate the efficiency of the RT

reactions (RT factor; conversion factor of RNA to cDNA). PCR amplification using universal 16S rRNA gene primers was tested on RNA extracts to confirm complete DNAse digestion.

AmoA genes and gene transcripts from ammonia oxidizing archaea (AOA) and bacteria (AOB) (β-proteobacteria) were quantified from extracted DNA and RNA (cDNA) according to Happel *et al.* (2018) using a BioRad ddPCR system and the primer sets Arch-amoAF (5 -STAATGGTCTGGCTTAGACG-3) and Arch-amoAR (5 -GCGGCCATCCATCTGTATGT) (Francis *et al.,* 2005), and amoA-1F (5-GGGGTTTCTACTGGTGGT-3) and amoA-2R (5-CCCCTCKGSAAAGCCTTCTTC-3) (Rotthauwe *et al.,* 1997), respectively. Each reaction mixture (25 µl) consisted of 10 µl Evagreen ddPCR mix (Bio-Rad), 200 nM of each primer, BSA (0.5 g μl^{-1}) and ca. 20 ng of template. The mixture was loaded with 70 µl Evagreen droplet generation oil into a droplet generator. PCR was performed in a T100 thermal cycler using a profile of 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 60°C (AOA) or 53°C (AOB) for 60 s, 1 cycle of 98°C for 10 min, and ending at 4°C. Droplets were read on the droplet reader and data analyzed using the QuantaSoft software (Bio-Rad). Quantification was presented as the number of target molecules per µl of PCR mixture and converted to copy number per liter seawater using the volume of water filtered. RNA sample quantifications were corrected using the RT factor of each sample. Equal extraction efficiencies for all samples are assumed.

Statistical analysis

To compare the bacterial communities, the taxonomic richness (calculated as abundance-based coverage estimator (ACE)) and the Shannon diversity index were estimated (Markussen *et al.,* 2018). Significant differences ($P<0.05$) between bacterial abundance and

192 production data were tested using Dunnett´s test (Markussen *et al.,* 2018). Raw counts of
193 EC/PFAM/COGs were normalized to counts per million (cpm) using EdgeR (Robinson *et al.,* 2010)
194 to test for significant differences in COG abundances between control samples and treatments.
195 Only COGs with an FDR <0.05 and p<0.005 were considered significant. Generalized linear models
196 (GLM) were investigated to test for significant correlations between the relative abundances of
197 EC/COG/PFAMs and environmental parameters using the mvabund (Wang *et al.,* 2012) package in
198 R. 16S rRNA gene OTU count data were normalized using DESeq2 (Love *et al.,* 2014) and principal
199 component analysis (PCA) was done using the R package. ANOSIM was done in R to test if
200 community composition differed between sites. ANOVA was used to test if *amoA* gene and
201 transcript abundances differed between treatments. Pearsons product moment coefficients were
202 used to test for correlations between environmental parameters and *amoA* gene and transcript
203 abundances.

204

205 ## Results and discussion

206 *The effect of agricultural and humic river water DOM on microbial communities*

207 In both experiments, there was a general increase in concentrations of DOC, DON, ammonium
208 (NH_4^+) and nitrate (NO_3^-) in the DOMhum and DOMagri treatments relative to the controls. In
209 particular high DON (104 µM) and NO_3 (62 µM) concentrations were found in the DOMagri
210 treatment in Exp I, whereas both river treatments had high DOC concentrations (711 and 756 µM,
211 respectively; Table 1) in Exp II.

212 Bacterial production (BP) (Fig. 1A, C) and abundance (BA) (Fig. 1B, D) increased
213 significantly over time in both experiments, but with large differences between the experiments.

BA (both experiments) and BP (Exp II) were significantly increased in both the DOMagri and DOMhum treatments compared with the controls, while BP was not significantly stimulated by either of the treatments in Exp I. The observed growth responses were anticipated to be accompanied by community dynamics mirrored in composition (16S rRNA genes) and function (composition and transcription of N cycling genes), based on earlier studies reporting that DOM can shape bacterioplankton community composition (McCarren *et al.,* 2010; Landa *et al.,* 2015; Traving *et al*., 2017). However, a PCA revealed that community composition at the end of the experiments clustered into site rather than treatment (Fig. 2A). Moreover, the community composition differed significantly between Exp I and II (ANOSIM, r^2=0.44, p<0.001) but not between treatments (ANOSIM, Exp I+II: r^2=0.04, p=0.88, Exp I: r^2=0.10, p=0.69, Exp II: r^2=0.49, p=0.09). Similarly, Shannon diversity (r^2 = 0.67, p < 0.001) and taxonomic richness was significantly higher in Exp II compared to Exp I (r^2 = 0.24, p < 0.001); however, no significant differences in alpha-diversity were observed between treatments from the same experiment. Hence, the changes in bacterial growth were only accompanied by limited shifts in community composition – and this was observed in both examined environments with marked differences in local community composition.

At the phylum level, e.g. γ-proteobacteria were significantly over-represented in the DOMagri treatment in Exp I and in both DOMagri and DOMhum in Exp II (Fig. 3A). In Exp II, e.g. β-proteobacteria were stimulated in the DOMhum treatment relative to the controls. Such stimulation of β-proteobacteria by DOM has previously been observed for Baltic bacterioplankton (Kisand and Wikner, 2003; Traving *et al.,* 2017). At the family level, there were several responses within Proteobacteria (Fig. 3B); e.g. Alteromonadaceae was more abundant in the DOMagri treatment relative to control in Exp I (DOMagri: 58%; Control: 25%). Within one of the abundant

237 groups, Bacteroidetes, responses were limited (Fig. 3C). Hence, some differences were observed in
238 composition between treatments (Fig. 3), but overall changes were considerably less than the
239 difference between environments (Fig. 2). There are examples of resistant microbial composition
240 withstanding disturbance (e.g. Bowen *et al.,* 2011); however, it may also be that DOC
241 manipulations, as in the current study, only select for some taxa whereas the relative abundance
242 of most taxa remain unchanged. Hence, it appears that overall community structure is a relatively
243 poor predictor of the bacterial growth response, as also suggested by others (Dinasquet *et al.,*
244 2013).

245

246 *Relative abundance of nitrogen cycling genes*

247 It has been suggested that the key level at which to address the assembly and structure of
248 bacterial communities is not taxonomy but rather the more functional level of genes (Burke *et al.,*
249 2011; Krause *et al.,* 2014). Moreover, since N availability affects N cycling genes (e.g. Zhang *et al.,*
250 2013), we hypothesized that the high N concentrations in the added river water would elicit
251 extensive and differential responses in the relative abundance of N cycling genes at the two sites.
252 Nevertheless, as in the compositional analysis, a PCA of the relative abundance of N cycling genes
253 (EC/EggNOG/PFAM; see Table S1) revealed a clustering according to site (ANOSIM, r^2=0.1944,
254 P=0.003) rather than treatment (ANOSIM, Exp I+II: r^2=0.06, p=0.62, Exp I: r^2=0.15, p=0.50, Exp II:
255 r^2=0.51, p=0.037) (Fig. 2B). Generalized linear models (GLM) showed that the relative abundances
256 of all N cycling genes did not correlate with any environmental parameters in Exp I. In Exp. II, on
257 the other hand, there were significant correlations with NH_4 (LR=1438.8, p=0.026), DOC
258 (LR=1592.3, p=0.011), DON (LR=1508.8, p=0.012) and treatment (LR=2665.3, p=0.018). This
259 suggests that while initial natural communities had a significant impact on the functional response

to the river water amendments, addition of river water with a high DOC to DON ratio in Exp. II (Table 1) also affected the abundance of N cycling genes.

Despite that the community analysis of N cycling genes did not reveal a clustering according to treatment (Fig. 2B), the relative abundance of some specific genes did differ significantly between controls and treatments (see below). However, in line with the above GLM results, more were over- or under- represented in Exp II than Exp I (Fig. 4). This suggests that a universal response (across sites) in N cycling genes due to treatment alone was not the case, but rather that the community of the northern Baltic Sea (Exp II; Storfjärden) was more responsive than that of the southern Baltic Sea (Exp I; Øresund). Reasons for this may include multiple site characteristics or seasonality (sampling in April vs. July); however, it is noteworthy that DOC levels naturally, and in our experiment, are highest in the northern Baltic (Table 1) (Sandberg *et al.,* 2004; Rowe *et al.,* 2018). Bacterioplankton in this environment may, therefore, be particularly adapted and responsive to pulses of riverine DOM. In addition, the higher diversity and taxonomic richness of Storfjärden could possibly have benefited the responsiveness of this community.

In Exp I, a N_2 fixation related gene (*nifB*, COG0535) and a nitrous oxide reduction gene (*nosZ*, PF13473) were over-represented in the DOMhum treatment relative to the control. Further, both ammonia transporters (COG0004, PF00909) and nitrite/nitrate reductases (PF07732, PF00394, PF04879) were under-represented (Fig. 4A). The DOMagri treatment did not have any significant effect on the relative abundance of N related genes (Fig. 4B). In Exp II, several EC/COG/PFAMs (DOMhum: 20; DOMagri: 13) differed significantly in relative abundance between treatments and controls (Fig. 4C,D). Among these, some of the N_2 fixation related genes were significantly over-represented in the DOMhum treatment (COG1348, PF00142, PF00148, EC

1.18.6.1) while one was under-represented (PF04055) (Fig. 4C). Most ammonia and nitrite/nitrate transporters were under-represented (PF00909, COG0004, PF07690) along with two urease genes (PF07969, PF01979). Both nitrate reductase (EC 1.7.99.4) and nitrous oxide reductases (COG4263, EC 1.7.2.4) were over-represented. In the DOMagri treatment, a single N_2 fixation gene was under-represented (COG0535) while both nitrite/nitrate transporters (COG2223) and nitrous oxide reductases (COG4263, EC 1.7.2.4, EC 1.7.1.14) were over-represented (Fig. 4D).

While there were only few responses in N related genes to the DOMagri treatments, there were several overlaps in the response to the DOMhum treatment between the two experiments. The under-representation of ammonia channel protein AmtB and over-representation of N_2 fixation genes in the DOMhum treatments, both point to possible N limitation (Carini *et al.,* 2018). There were, however, no indications of N limitation when looking at the N:P ratios. The N:P ratios (calculated as $(NH_4+NO_3)/PO_4$) were highest in the DOMagri treatments in Exp. I and in the DOMhum treatment in Exp. II. Further, the over-representations of nitrate reductases (Exp I) and nitrous oxide reductases (both experiments) could indicate a promotion of some steps of the denitrification pathway by the addition of DOMhum. Denitrification in the Baltic Sea is known from anoxic zones of the water column (Dalsgaard *et al.,* 2013) and from sediments (Silvennoinen *et al.,* 2007).

Abundance and activity of ammonia oxidizing archaea (AOA) and bacteria (AOB)

To quantitatively assess the impact of the treatments on functional gene abundance and transcription, digital droplet PCR (ddPCR) was used to enumerate *amoA* genes and transcripts of AOA and AOB in each experiment initially, after 44-45 h, and at the end of each experiment (Fig. 5). The ddPCR method was chosen because of its documented ability to quantify *amoA* genes from

Baltic Sea waters (Happel *et al.* 2018). For principles and details of the ddPCR methodology, please see the recent study by Happel and co-authors (2018).

Although *amoA* gene and transcript abundances of both AOA and AOB were dynamic over time, there was no significant treatment effect on *amoA* abundances. There were, however, significant differences between the *amoA* transcript abundances of DOMhum and control treatments in Exp I for both AOA (one-way ANOVA; F=11.7, p=0.011 and one-way ANOVA on ranks; Q=2.6, p=0.014) and AOB (one-way ANOVA on ranks; Q=2.32, p=0.024 and Q=2.55, P=0.032, respectively) at the beginning of the experiment. In both experiments, we found abundances of AOA ($3.9 \times 10^3 – 7.3 \times 10^4$ copies L^{-1}) and AOB ($4.2 \times 10^3 – 5.4 \times 10^4$ copies L^{-1}) in similar ranges, whereas *amoA* gene transcription was dominated by AOB (up to 1.5×10^9 copies L^{-1} for AOB and 1.5×10^8 copies L^{-1} for AOA). Concentrations of AOA and AOB were remarkably low compared to coastal environments (Beman *et al.,* 2008, Hollibaugh *et al.,* 2011), but similar to the Yangtze River estuary (Zhang *et al.,* 2014) and to our previous study on ammonia oxidizers in the south and north of the Baltic Sea (Happel *et al*., 2018). Moreover, the dominance of *amoA* transcripts from AOB also matches our previous Baltic Sea study (Happel *et al.,* 2018). Surprisingly, there was no significant stimulation of *amoA* gene abundances from AOA or AOB by the addition of riverine DOM in either experiment and *amoA* gene transcription was in fact reduced in the DOMhum treatments relative to controls. This could be interpreted as a sign of ammonia limitation (Carini *et al.,* 2018), however, since *amoA* gene transcription from AOB was negatively correlated with DOC in Exp I (Pearson correlation, r=-0.34, p=0.04), we speculate that ammonia oxidizers were hampered by the introduction of riverine DOC – potentially the sudden availability of labile riverine carbon is at odds with the chemolithoautotrophic life strategy of ammonia oxidizers (Strauss and Lamberti, 2000; Strauss *et al.,* 2002).

Concluding remarks

Despite that the addition of river water caused several folds increase in bacterial growth in both the Øresund and Storfjärden experiments, only specific sub-populations and N cycling processes were affected by the treatments, whereas overall community composition and the collective pool of examined N cycling genes remained relatively unaffected. This may support the notion that many bacterioplankton species are generalists and less responsive to transient changes in the DOC pool (Mou *et al.,* 2008), and that the linkage between identity and specialized DOC utilization is valid only within some phyla or among specific sub-populations (Dinasquet *et al.,* 2013). Interestingly, treatment effects on nitrification were only observed at the transcription level, and not the gene level. This observation underlines that functional responses in key N cycling processes in bacterioplankton may not always be accompanied by selection affecting community composition. Our study included several experimental variables like seasons and river inocula which prevents firm comparisons between the two localities; south and north in the Baltic Sea. Nevertheless, the higher responsiveness of the community in Storfjärden to riverine DOM sources is noteworthy. If coupled with the projected future increases in precipitation and outlet of allochthonous DOM (Andersson *et al.,* 2015), we speculate that the coastal zones in the Northern Baltic Sea will undergo more dramatic future changes in N cycling regimes than the communities in the Øresund. However, focused studies are needed in order to validate this hypothesis.

Acknowledgements

This work resulted from the BONUS Blueprint project supported by BONUS (Art 185), funded

jointly by the EU and the Danish Council for Independent Research, Estonian Research Council, Swedish Research Council FORMAS, and Academy of Finland. We kindly thank Tvärminne Zoological Station, University of Helsinki, and its personnel for providing infrastructural support. The authors declare no conflict of interest.

References

Alneberg, J., Sundh, J., Bennke, C., Beier, S., Lundin, D., Hugerth, L.W., Pinhassi, J., Kisand, V., Riemann, L., Jürgens, K., Labrenz, M., Andersson, A.F. (2018) BARM and BalticMicrobeDB, a reference metagenome and interface to meta-omic data for the Baltic Sea. *Scientific Data* 5:180146

Andersson, A., Meier, H.E.M., Ripszam, M., Rowe, O., Wikner, J., Haglund, P. et al. (2015) Projected future climate change and Baltic Sea ecosystem management. *Ambio* **44**: S345-S356.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A., and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Mar Ecol Prog Ser* **10**: 257-263.

Beman, J.M., Popp, B.N., and Francis, C.A. (2008) Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. The ISME Journal 2: 429-441.

Bernes, C. (2005) Change Beneath the Surface. An In-Depth Look at Sweden's Marine Environment. *Fälth & Hässler, Värnamo, Sweden Monitor* **19**: 38-47.

Bowen, J.L., Ward, B.B., Morrison, H.G., Hobbie, J.E., Valiela, I., Deegan, L.A., and Sogin, M.L. (2011) Microbial community composition in sediments resists perturbation by nutrient enrichment. *The ISME Journal* **5**: 1540-1548.

372 Bristow, L.A., Mohr, W., Ahmerkamp, S., and Kuypers, M.M.M. (2017) Nutrients that limit growth
373 in the ocean. *Current Biology* **27**: R474-R478.
374 Bronk, D.A., See, J.H., Bradley, P., and Killberg, L. (2007) DON as a source of bioavailable nitrogen
375 for phytoplankton. *Biogeosciences* **4**: 283-296.
376 Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., and Thomas, T. (2011) Bacterial community
377 assembly based on functional genes rather than species. *Proc Natl Acad Sci U S A* **108**: 14288-
378 14293.
379 Carini, P., Dupont, C.L., and Santoro, A.E. (2018) Patterns of thaumarchaeal gene transcription in
380 culture and diverse marine environments. *Environ Microbiol*. Doi:10.1111/1462-2920.14107.
381 Dalsgaard, T., De Brabandere, L., and Hall, P.O.J. (2013) Denitrification in the water column of the
382 central Baltic Sea. *Geochimica et Cosmochimica Acta* **106**: 247-260.
383 Dinasquet, J., Kragh, T., Schroter, M.L., Sondergaard, M., and Riemann, L. (2013) Functional and
384 compositional succession of bacterioplankton in response to a gradient in bioavailable dissolved
385 organic carbon. *Environ Microbiol* **15**: 2616-2628.
386 Falkowski, P.G., Fenchel, T., and Delong, E.F. (2008) The microbial engines that drive Earth's
387 biogeochemical cycles. *Science* **320**: 1034-1039.
388 Finn, R.D., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Mistry, J., Mitchell, A.L. et al. (2016) The Pfam
389 protein families database: towards a more sustainable future. *Nucleic Acids Res* **44**: D279-285.
390 Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., and Oakley, B.B. (2005) Ubiquity and
391 diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings*
392 *of the National Academy of Sciences of the United States of America* **102**: 14683-14688.

393 Fuhrman, J.A., and Azam, F. (1982) Thymidine incorporation as a measure of heterotrophic
394 bacterioplankton production in marine surface waters - evaluation and field results. *Marine*
395 *Biology* 66: 109-120.

396 Galperin, M.Y., Makarova, K.S., Wolf, Y.I., and Koonin, E.V. (2015) Expanded microbial genome
397 coverage and improved protein family annotation in the COG database. *Nucleic Acids Res* **43**:
398 D261-269.

399 Glibert, P.M., Wilkerson, F.P., Dugdale, R.C., Raven, J.A., Dupont, C.L., Leavitt, P.R. et al. (2016)
400 Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and
401 implications for productivity and community composition, with emphasis on nitrogen-enriched
402 conditions. *Limnology and Oceanography* **61**: 165-197.

403 Happel, E., Bartl, I., Voss, M. and Riemann, L. (2018) Extensive nitrification and abundance of
404 active bacterial ammonia oxidizers in two contrasting coastal systems of the Baltic Sea.
405 *Environmental Microbiology* 20 (8): 2913-2926.

406 Herlemann, D.P., Labrenz, M., Jurgens, K., Bertilsson, S., Waniek, J.J., and Andersson, A.F. (2011)
407 Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. The
408 ISME Journal **5**: 1571-1579.

409 Hietanen, S., Jäntti, H., Buizert, C., Jürgens, K., Labrenz, M., Voss, M., and Kuparinen, J. (2012)
410 Hypoxia and nitrogen processing in the Baltic Sea water column. *Limnology and Oceanography* **57**:
411 325-337.

412 Hollibaugh, J.T., Gifford, S., Sharma, S., Bano, N., and Moran, M.A. (2011) Metatranscriptomic
413 analysis of ammonia-oxidizing organisms in an estuarine bacterioplankton assemblage. The ISME
414 Journal 5: 866-878.

Holmes, R.M., Aminot, A., Kérouel, R., Hooker, B.A., and Peterson, B.J. (1999) A simple and precise method for measuring ammonium in marine and freshwater ecosystems. *Canadian Journal of Fisheries and Aquatic Sciences* 56: 1801-1808.

Khodursky, A.B., Bernstein, J.A., Peter, B.J., Rhodius, V., Wendisch, V.F., and Zimmer, D.P. (2003) *Escherichia coli* spotted double-strand DNA microarrays. In Functional Genomics: Methods and Protocols. Brownstein, M.J., and Khodursky, A.B. (eds). Totowa, NJ: Humana Press, pp. 61-78.

Kisand, V., and Wikner, J. (2003) Combining culture-dependent and -independent methodologies for estimation of richness of estuarine bacterioplankton consuming riverine dissolved organic matter. *Applied and Environmental Microbiology* **69**: 3607-3616.

Knudsen-Leerbeck, H., Mantikci, M., Bentzon-Tilia, M., Traving, S.J., Riemann, L., Hansen, J.L.S., and Markager, S. (2017) Seasonal dynamics and bioavailability of dissolved organic matter in two contrasting temperate estuaries. *Biogeochemistry* **134**: 217-236.

Krause, S., Le Roux, X., Niklaus, P.A., Van Bodegom, P.M., Lennon, J.T., Bertilsson, S. et al. (2014) Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. *Frontiers in Microbiology* **5**: 1-10.

Landa, M., Blain, S., Christaki, U., Monchy, S., and Obernosterer, I. (2015) Shifts in bacterial community composition associated with increased carbon cycling in a mosaic of phytoplankton blooms. *The ISME Journal* **10**: 39.

Langmead, B., and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**: 357.

Li, W., Fu, L., Niu, B., Wu, S., and Wooley, J. (2012) Ultrafast clustering algorithms for metagenomic sequence analysis. *Briefings in Bioinformatics* 13: 656-668.

437 Love, M.I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion
438 for RNA-seq data with DESeq2. *Genome Biology* 15: 550.

439 Markussen, T., Happel, E.M., Teikari, J. E., Huchaiah, V., Alneberg, J., Andersson, A. F., Sivonen, K.,
440 Riemann, L., Middelboe, M., Kisand, V. (2018) Coupling biogeochemical process rates and
441 metagenomic blueprints of coastal bacterial assemblages in the context of environmental change.
442 *Environmental Microbiology* **20(8)**: 3083-3099

443 Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads.
444 *EMBnetjournal* **17**: 10.

445 McCarren, J., Becker, J.W., Repeta, D.J., Shi, Y., Young, C.R., Malmstrom, R.R. et al. (2010)
446 Microbial community transcriptomes reveal microbes and metabolic pathways associated with
447 dissolved organic matter turnover in the sea. *Proceedings of the National Academy of Sciences* U S
448 A **107**: 16420-16427.

449 Mou, X., Sun, S., Edwards, R.A., Hodson, R.E., and Moran, M.A. (2008) Bacterial carbon processing
450 by generalist species in the coastal ocean. *Nature* **451**: 708-711.

451 Murphy, J., and Riley, J.P. (1962) A modified single solution method for the determination of
452 phosphate in natural waters. *Analytica Chimica Acta* 27: 31-36.

453 Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glöckner, F.O. (2007)
454 SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence
455 data compatible with ARB. *Nucleic Acids Research* **35**: 7188-7196.

456 Quinlan, A.R. (2014) BEDTools: the Swiss-army tool for genome feature analysis. *Current Protocols*
457 *in Bioinformatics* **47**: 11.12.11-11.12.34.

458 Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010) edgeR: a Bioconductor package for
459 differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139-140.
460 Rönnberg, C., and Bonsdorff, E. (2004) Baltic Sea eutrophication: area-specific ecological
461 consequences. *Hydrobiologia* **514**: 227-241.
462 Rotthauwe, J.H., Witzel, K.P., and Liesack, W. (1997) The ammonia monooxygenase structural gene
463 *amo*A as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing
464 populations. *Applied and Environmental Microbiology* **63**: 4704-4712.
465 Rowe, O.F., Dinasquet, J., Paczkowska, J., Figueroa, D., Riemann, L., and Andersson, A. (2018)
466 Major differences in dissolved organic matter characteristics and bacterial processing over an
467 extensive brackish water gradient, the Baltic Sea. *Marine Chemistry* **202**: 27-36.
468 Paulsen, M.L., Nielsen, S.E.B., Müller, O., Møller, E.F., Stedmon, C.A., Juul-Pedersen, T. et al. (2017)
469 Carbon bioavailability in a high arctic fjord influenced by glacial meltwater, NE Greenland.
470 *Frontiers in Marine Science* 4.
471 Ryther, J.H., and Dunstan, W.M. (1971) Nitrogen, phosphorus, and eutrophication in the coastal
472 marine environment. *Science* **171**: 1008-1013.
473 Sandberg, J., Andersson, A., Johansson, S., and Wikner, J. (2004) Pelagic food web structure and
474 carbon budget in the northern Baltic Sea potential importance of terrigenous carbon. *Marine*
475 *Ecology Progress Series* **268**: 13-29.
476 Seitzinger, S.P., Harrison, J.A., Dumont, E., Beusen, A.H.W., and Bouwman, A.F. (2005) Sources and
477 delivery of carbon, nitrogen, and phosphorus to the coastal zone: An overview of Global Nutrient
478 Export from Watersheds (NEWS) models and their application. *Global Biogeochemical Cycles* **19:**
479 1-11.

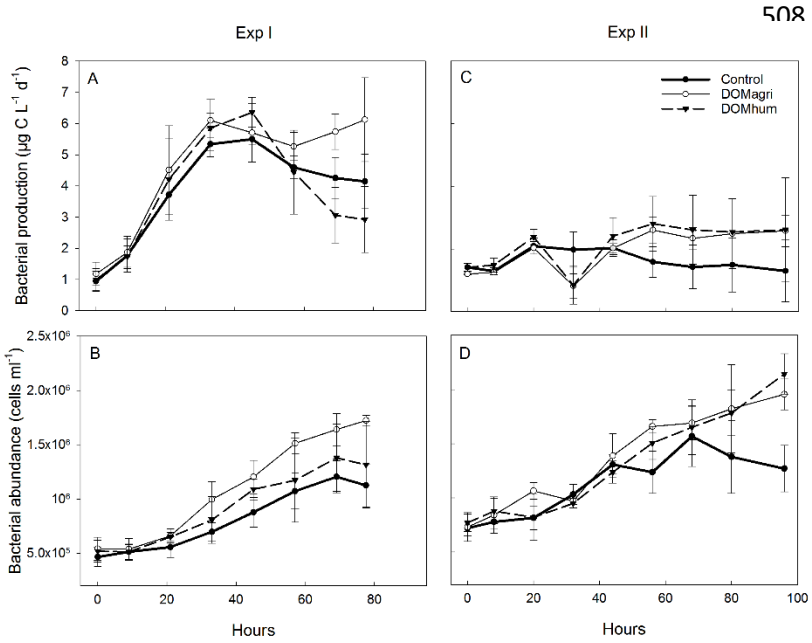
480 Silvennoinen, H., Hietanen, S., Liikanen, A., Stange, C.F., Russow, R., Kuparinen, J., and
481 Martikainen, P.J. (2007) Denitrification in the river estuaries of the northern Baltic Sea. *Ambio* **36**:
482 134-140.
483 Strauss, E.A., and Lamberti, G.A. (2000) Regulation of nitrification in aquatic sediments by organic
484 carbon. *Limnology and Oceanography* **45**: 1854-1859.
485 Strauss, E.A., Mitchell, N.L., and Lamberti, G.A. (2002) Factors regulating nitrification in aquatic
486 sediments: effects of organic carbon, nitrogen availability, and pH. *Canadian Journal of Fisheries*
487 *and Aquatic Sciences* **59**: 554-563.
488 Traving, S.J., Rowe, O., Jakobsen, N.M., Sorensen, H., Dinasquet, J., Stedmon, C.A. et al. (2017) The
489 Effect of increased loads of dissolved organic matter on estuarine microbial community
490 composition and function. *Frontiers in Microbiol* **8**: 351.
491 Wang, Y., Naumann, U., Wright, S.T., and Warton, D.I. (2012) mvabund– an R package for model-
492 based analysis of multivariate abundance data. *Methods in Ecology and Evolution* 3: 471-474.
493 Wood, E.D., Armstrong, F.A.J., and Richards, F.A. (2009) Determination of nitrate in sea water by
494 cadmium-copper reduction to nitrite. *Journal of the Marine Biological Association of the United*
495 *Kingdom* 47: 23-31.
496 Xu, H., Luo, X., Qian, J., Pang, X., Song, J., Qian, G. et al. (2012) FastUniq: A fast de novo duplicates
497 removal tool for paired short reads. *PLOS ONE* **7**: e52249.
498 Zehr, J.P., and Ward, B.B. (2002) Nitrogen cycling in the ocean: new perspectives on processes and
499 paradigms. *Appl Environ Microbiol* **68**: 1015-1024.

500 Zhang, X., Liu, W., Schloter, M., Zhang, G., Chen, Q., Huang, J. et al. (2013) Response of the
501 abundance of key soil microbial nitrogen-cycling genes to multi-factorial global changes. *PLOS ONE*
502 **8**: e76500.

503 Zhang, Y., Xie, X., Jiao, N., Hsiao, S.S.Y., and Kao, S.J. (2014) Diversity and distribution of *amo*A-type
504 nitrifying and *nir*S-type denitrifying microbial communities in the Yangtze River estuary.
505 *Biogeosciences* 11: 2131-2145.

506

507 Table and figure legends



515

516 Figure 1. Bacterial abundance and bacterial production during Exp I (Øresund) (A, B) and Exp II
517 (Storfjärden) (C, D) for the controls and the agriculture (DOMagri) and the humic (DOMhum) river
518 water treatments. Data are adapted from Markussen *et al.* (2018). Error bars indicated SD of
519 biological triplicates (6 replicates in control samples).

520

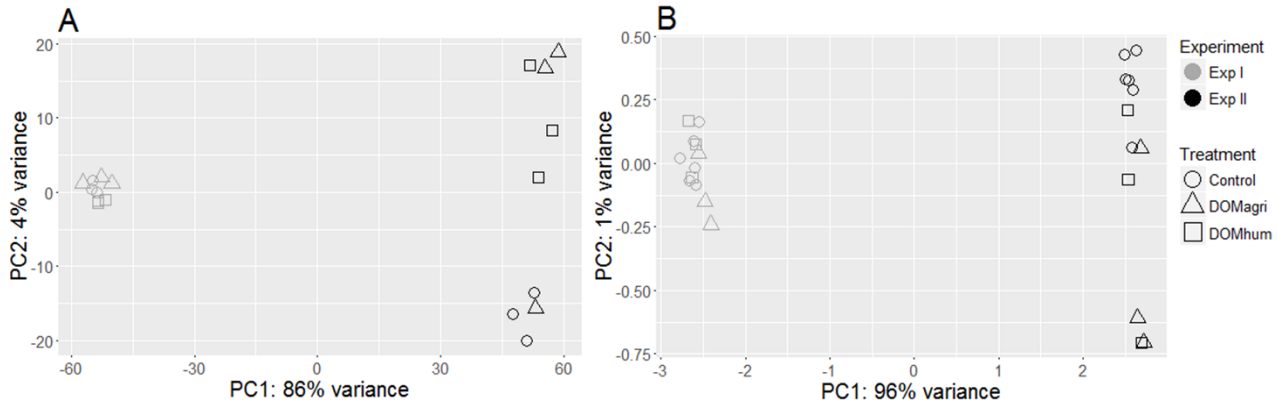
521

522

523

524

525



526

527 Figure 2. Principal component analysis (PCA) of community composition (based on 16S rRNA
528 sequencing; A) and nitrogen related genes (EC/eggNOG/PFAM) (metagenomics sequencing; B) at
529 the end of Exp I (Øresund) and Exp II (Storfjärden) for the controls and the agriculture (DOMagri)
530 and humic (DOMhum) river water treatments. For separate PCA of each experiment; see Figure
531 S2.

532

533

534

535

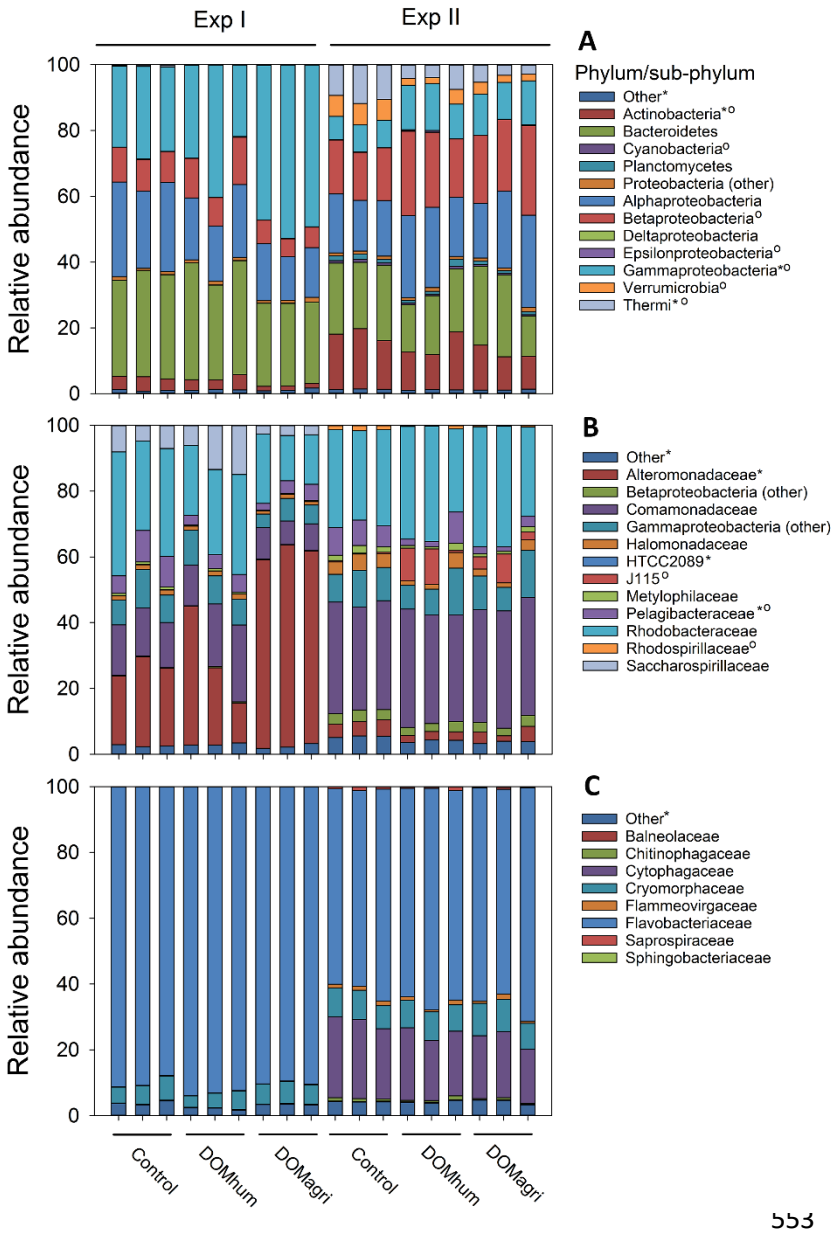
536

537

538

539

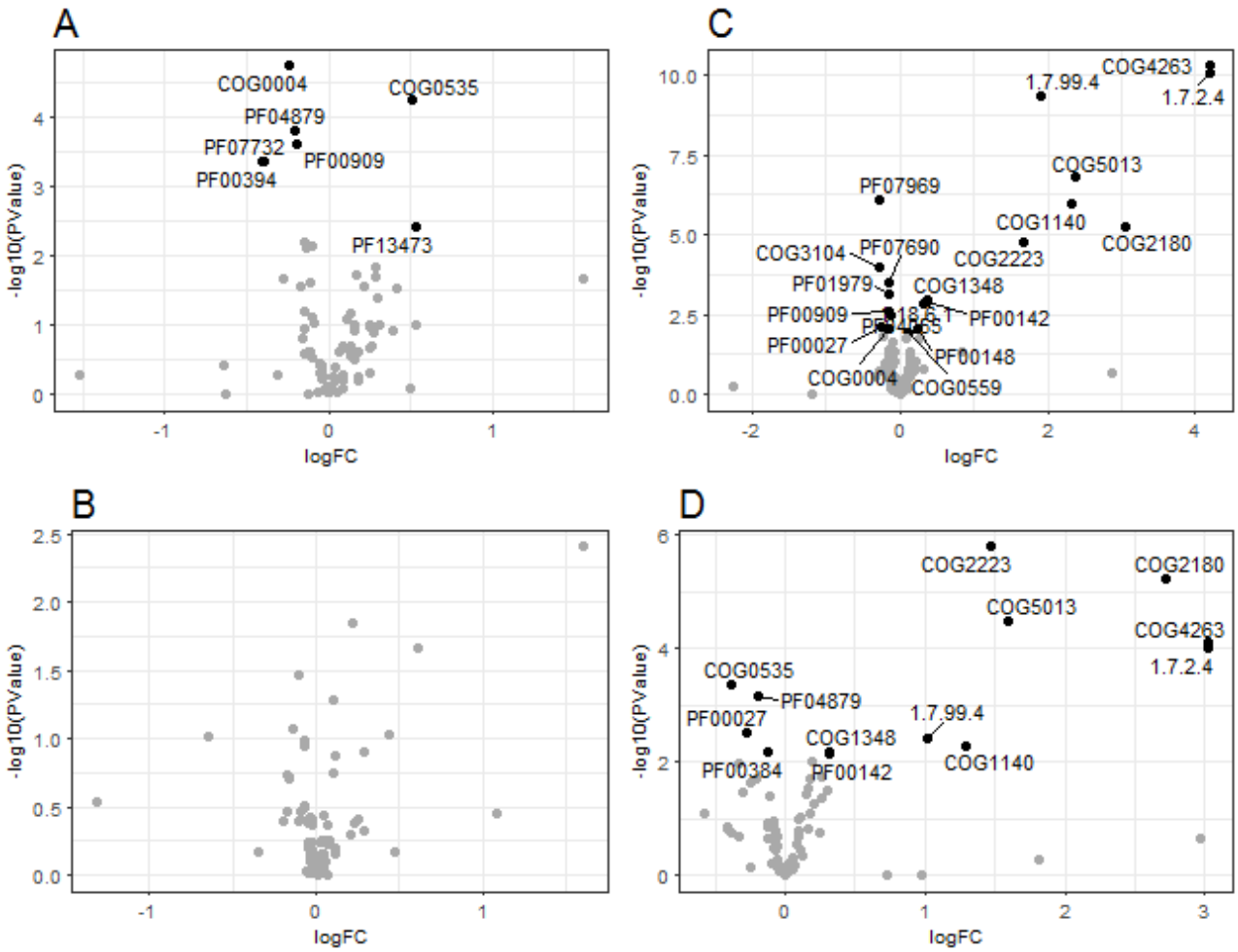
540



553

554 Figure 3. Relative abundance of phyla/sub-phyla (A), families within Proteobacteria (B) and
555 Bacteriodetes (C) at the end of Exp I (Øresund) and II (Storfjärden) for the controls and the
556 agriculture river water (DOMagri) and the humic river water (DOMhum) treatments. Significant
557 differential abundances for each group between treatments were tested using EdgeR and
558 indicated for Exp I (*) and Exp II (°).

559



560

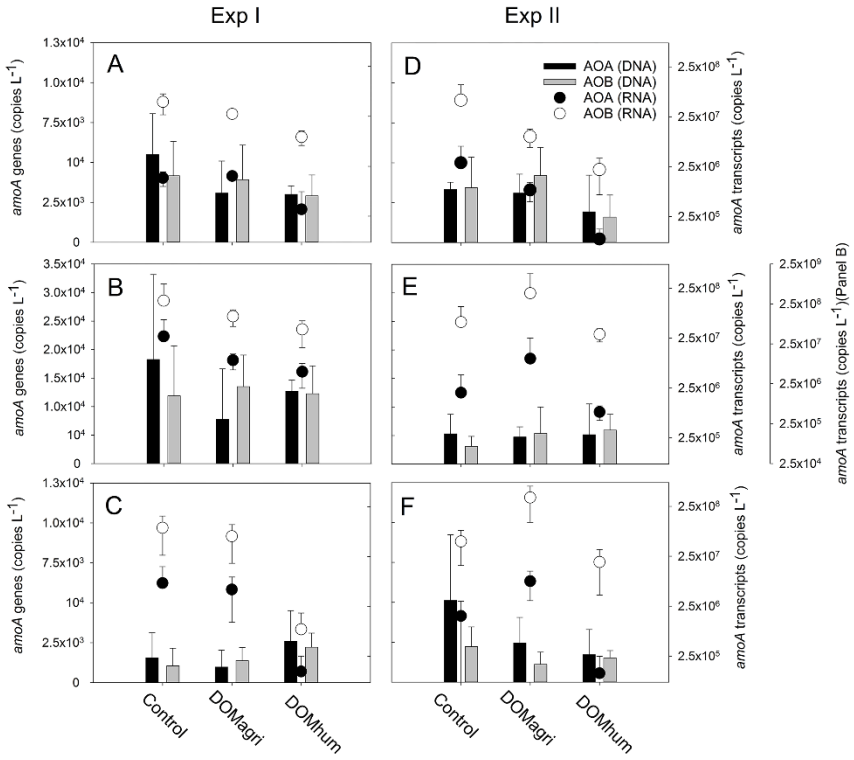
561 Figure 4. Volcano plots showing up- and down- represented EC/PFAM/COGs in Exp I (A, B) and Exp
562 II (C, D) for the controls and the humic (DOMhum)(A, C) and the agriculture (DOMagri) (B, D) river
563 water treatments. Significantly differentially abundant EC/PFAM/COGs are marked with black
564 dots.

565

566

567

568



577 Figure 5. Archaeal (AOA) and bacterial (AOB) *amoA* gene and transcript abundances at the start of
578 the experiments (A,D), after 44-45 hours (B,E) and at the end of the experiments (C,F) for Exp I
579 (left) and Exp II (right) for the controls and the agriculture (DOMagri) and humic (DOMhum) river
580 water treatments. Note the different scales in (B). Error bars indicated SD of biological triplicates
581 (6 replicates for control samples).

582

583 Table 1

584

	Exp I (Øresund, Southern Baltic Sea)			**Exp II** (Storfjärden, Northern Baltic Sea)		
Treatment	Control	DOMhum	DOMagri	Control	DOMhum	DOMagri
DOC (µM)	381.50 (13.66)	552.67 (51.43)	481.67 (78.23)	555.50 (80.77)	711.33 (66.98)	756.00 (59.43)
DON (µM)	47.37 (0.37)	51.97 (1.34)	103.57 (15.97)	48.05 (2.16)	51.77 (1.27)	57.10 (0.90)
NH_4 (µM)	0.57 (0.02)	0.94 (0.12)	1.03 (0.04)	0.35 (0.03)	0.54 (0.03)	1.12 (0.01)
PO_4 (µM)	0.06 (0.03)	0.20 (0.02)	0.19 (0.08)	0.09 (0.04)	0.08 (0.02)	0.09 (0.10)
NO_3 (µM)	1.74 (0.19)	3.63 (0.66)	61.87 (9.35)	0.27 (0.08)	2.60 (0.66)	0.56 (0.08)

585

586 Table 1. Concentrations of dissolved organic carbon (DOC), dissolved organic nitrogen (DON)
587 ammonium (NH_4), phosphate (PO_4) and nitrate (NO_3) in the treatments at the beginning of the
588 experiments for the control, the agriculture river water treatment (DOMagri) and the humic river
589 water treatment (DOMhum). Standard deviations in brackets.

590

591